Iso-1-cytochromes c having lysine 32 replaced by leucine, glutamine, tyrosine, and tryptophan were prepared from strains of bakers' yeast, Saccharomyces cerevisiae, and chemically blocked at cysteine 107 with methyl methanethiosulfonate to prevent denaturation. These modified ferricytochromes c were guanidine denatured and the unfolding thermodynamics were determined by circular dichroism and fluorescence measurements. Thermal unfolding was also monitored by absorbance measurements. The guanidine denaturation midpoints for the altered proteins are smaller than the wild type, while the orders of stability from unfolding free energy changes are: Lys-32 (wild type) ≈ Leu-32 ≈ Gln-32 (circular dichroism), > Gln-32 (fluorescence) > Tyr-32 ≈ Trp-32. Midpoints and differences in free energy changes for thermal unfolding parallel the fluorescence free energy changes for guanidine-induced unfolding. Thus, the blocked Leu-32 and Lys-32 proteins are equally stable with respect to both chemical and thermal denaturation. The reported data indicate that single replacements may significantly modify protein stability, and that substitution for an evolutionarily retained residue in normal cytochrome c structures does not always destabilize the protein. In addition, in vitro thermal stabilities approximately correlate with in vivo specific activities.

Proteins having single amino acid replacements are invaluable for investigating structure-function and stability relationships. Iso-1-cytochrome c from bakers' yeast, Saccharomyces cerevisiae, is a particularly useful model compound for such studies for the following reasons: the genetics of the iso-1 system have been well characterized (Sherman and Stewart, 1978), isolation of mutant strains (Sherman et al., 1974) and oligonucleotide-directed mutagenesis are routinely accomplished, many altered forms of iso-1 are available (Hampsey et al., 1986; Hill and Pettigrew, 1975), and the proteins having lysine 32 replaced by glutamine, leucine, tryptophan, or tyrosine, respectively, replace lysine at position 77. Also, iso-1 with arginine 77 has in vitro catalytic activity with its physiological partners at least as great as the wild type protein (Holzschu et al., 1987). When the conserved proline 76 in iso-1 was replaced with leucine, isoleucine, serine, threonine, or valine, the resulting in vivo activities ranged from 0 to 90% of the normal value. In vitro thermodynamic stabilities of modified iso-1-cytochromes c having the latter four substitutions were measured by guanidine-induced denaturation and correlated with in vivo growth rates (Ramdas et al., 1986). These replacements also changed the rate of the fast kinetic phase in folding the altered proteins (Ramdas and Nall, 1986).

The one crystallographic structure has been determined to a resolution of 1.8 Å (Louis et al., 1988). In addition, 23 evolutionarily conserved residues in 91 species have been identified (Hampsey et al., 1986; Cutler et al., 1987). Although these invariant residues are undoubtedly conserved through evolution because they serve a function, the degree of importance can not be assessed simply from the consensus sequence. Furthermore, evolutionary invariance does not necessarily imply functional invariance. The extent to which a residue is required can be experimentally determined by investigating mutants in vivo and the proteins in vitro. For example, in vivo growth rates are normal or near normal for yeast strains whose iso-1 has arginine replacing the invariant lysine at position 77. Also, iso-1 with arginine 77 has in vitro catalytic activity with its physiological partners at least as great as the wild type protein (Holzschu et al., 1987). When the conserved serine 76 in iso-1 was replaced with leucine, isoleucine, serine, threonine, or valine, the resulting in vivo activities ranged from 0 to 90% of the normal value. In vitro thermodynamic stabilities of modified iso-1-cytochromes c having the latter four substitutions were measured by guanidine-induced denaturation and correlated with in vivo growth rates (Ramdas et al., 1986). These replacements also changed the rate of the fast kinetic phase in folding the altered proteins (Ramdas and Nall, 1986). Lysine is found at position 32 in 88 of 92 known cytochrome c amino acid sequences (Hampsey et al., 1986; Cutler et al., 1987). The four primary sequences lacking Lys-32 form a class of abnormal cytochrome c structures: cytochromes c from Euglena gracilis (Pettigrew et al., 1975), Crithidia fasciculata (Hill and Pettigrew, 1975), Crithidia oncopelti (Pettigrew et al., 1975), and Tetrahymena pyriformis (Tarr and Fitch, 1976) have Ser, Gly, Gly, and Thr, respectively, at position 32 (using the iso-1 numbering system). In addition, the first three of these protozoan proteins have only a single Cys-22 thioether linkage to the heme vinyl group (Pettigrew et al., 1975; Hill and Pettigrew, 1975). Deletions also occur at positions 27–28 and 30 in the Tetrahymena (Tarr and Fitch, 1976) and Euglena (Pettigrew et al., 1975) cytochromes c, respectively.

Thus, within the class of 88 typical cytochrome c structures, Lys-32 in S. cerevisiae is evolutionarily retained. Mutant yeast strains having substitutions at this site exhibit decreased growth rates on lactate medium relative to the wild type, while in vitro steady state kinetic parameters for reactivity with cytochrome b5, cytochrome c peroxidase, and cytochrome oxidase are not substantially changed by mutation. (Das et al., 1988). We have measured the relative stabilities of iso-1 replaced at Lys-32 with glutamine, leucine, tryptophan, and typtophan. Each protein was chemically denatured with guanidine hydrochloride and the resulting transitions were followed with circular dichroism and fluorescence measurements.
ments. Absorbance measurements were also used to monitor thermal unfolding. To insure that dimerization through cysteine 107 disulfide linkages did not affect the unfolding and to permit thermal denaturations, thermodynamic quantities were determined using protein modified at Cys-107, methanethiol-sulfonate (MMTS) blocked protein. Further experiments a better understanding has emerged of how the stability of cytochrome c is both affected by residue substitution and contributes to evolutionary selection.

MATERIALS AND METHODS

Source of Chemical—All phosphate buffers were freshly prepared with J. T. Baker Chemical Co. Analyzed Reagent grade or EM Science salts and twice-distilled water.

Source of Proteins—The S. cerevisiae strains having altered residues at position 32 are described in Das et al. (1986). In brief, genetic tests demonstrated that a mutant lacking iso-1 had an amber UAG codon corresponding to position 32. Revertant strains coding for Leu-32 (UUA), Gin-32 (CAG), Tryp-32 (UUG), and Tyr-32 (UAU) were produced by single nucleotide changes of the nonsense UAG codon using UV irradiation (Gin-32 and ethyl sulfate (Leu-32, Tryp-32, Tyr-32), followed by isolation on lactate medium (Sherman et al., 1974). The mutant strains were then identified by cloning and DNA sequencing the altered iso-1 structural genes.

Wild type iso-1 was extracted from commercially available baker's yeast, recombinant Leu-32, an iso-1-1, and Cys-107, were obtained from strains grown in fermentors (New Brunswick Scientific, model SF-116) at 30 °C following the procedure of Sherman et al. (1986). The extractions were similar to that described in Sherman et al. (1986) except that: (i), the first batch of coarse Amberlite was stirred for 1 h before adding the second; (ii), (NH4)2SO4 and (NH4)2CO3 were removed by centrifugation before starting the gradient; (iii), after gel filtration, the iso-1 concentration and oxidation state were determined from an absorbance spectrum obtained with a Perkin-Elmer Lambda Array UV/Vis Spectrophotometer interfaced to an IBM Personal Computer. The printed spectra were later digitized with a Tektronix 4051 computer and 4062 Interactive Digital Plotter, and transferred to a magnetic tape for plotting on the same device. The absorbance versus wavelength data were normalized to the absorbance at 280 nm for both normal and mutant species (Margoliash and Frischwitz, 1959) and converted to extinction coefficients using ε590 = 106.1 × 106 liter/mol-cm, and plotted for comparison.

Chemical Denaturations—If the normal and altered iso-1-SCH3 proteins were not fully oxidized, KFe(CN)6 was added, followed by separation on a Sephadex G-25-80 column with 50 mM sodium phosphate, pH 7.2. Solutions for physical measurements were batch-prepared by diluting the protein stock solutions into mixtures of varying proportions of 4 M guanidine hydrochloride, pH 6.8 (Schwarz-Mann, ultrapure) and a stock buffer of 20 mM Tris (Sigma) and 40 mM NaCl, pH 7.2. Finally, guanidine concentrations were about 0.03 M.

Equilibrium unfolding of chemically blocked mutant and normal proteins with guanidine HCl concentrations ranging from 0 to 2 M was monitored at room temperature with circular dichroism and fluorescence measurements. Ellipticities were measured at 220 nm with a Jasco J-400 spectropolarimeter. CD spectra from 220 to 300 nm were also recorded at 0 and 2 mM guanidine HCl for these proteins. Fluorescence measurements were made with a Perkin-Elmer MPF-2A fluorometer (ratio mode) using an excitation wavelength of 292 nm.

The emission wavelengths centered in broad maxima were 292 nm (Leu-32-SCH3, Tryp-32-SCH3), and 362 nm (Leu-32-SCH3, Gin-32-SCH3) were plotted for both CD and fluorescence measurements.

Chemical Denaturation data were analyzed with a two-state equilibrium, $N \leftrightarrow D$, where $N$ and $D$ represent native and denatured states, respectively. For a structure-sensitive observable, the fraction of unfolded molecules at any guanidine concentration, $f_u$, is

\[ f_u = (X - X_D)/(X_N - X_D), \]

where $X$ and $X_D$ are limiting values of $X$ in the folded and unfolded states, respectively, while $X$ is its value at the guanidine concentration. The unfolding equilibrium constant, $K_u$, is then $f_u(1 - f_u)$.

The thermodynamic basis of solvent denaturation presented by Schellman (1975) states that for a two-state denaturation (using Schellman’s nomenclature): $K_u(C_3) = \ln K_u - \Delta G_u$, where $\Delta G_u$ is the unfolding free energy changes in the absence and presence of $C_3$ mol/liter, respectively. $f_u = (X - X_D)/(X_N - X_D)$ measures the extent of unfolding for these proteins:

\[ f_u = (X - X_D)/(X_N - X_D), \]

where $X$ and $X_D$ are limiting values of $X$ in the folded and unfolded states, respectively, while $X$ is its value at the guanidine concentration. The unfolding equilibrium constant, $K_u$, is then $f_u(1 - f_u)$.
they form dimers and higher order polymers that scatter light, thereby preventing observation of the thermally induced denaturation. Chemical modification therefore permits melting curve (absorbance versus temperature) experiments to be performed.

Fig. 1 illustrates the results of MMTS blocking for normal and Gln-32 iso-1. The unblocked proteins are mixtures primarily of monomers and some spontaneously formed dimers. Unmodified iso-1 reacts with CuSO₄, yielding mostly dimers along with an increase in higher order polymers according to the equation (Montonaga et al., 1965):

\[ 2\text{Cu}^{2+} + 2\text{S-H} \rightarrow \text{S-S} + 2\text{Cu}^{2+} + 2\text{H}^+ \]

This is shown by the bleaching of monomer bands and intensification of dimer bands in lanes A2 and B2. In contrast, iso-1-SCH₃ and Gln-32-SCH₃ are totally monomeric after chemical modification and remain so on treatment with CuSO₄ (lanes A4 and B4) or heat. MMTS-blocking thus guarantees that iso-1-cytochromes c will not dimerize under the experimental conditions reported here.**

**UV-visible Spectra—Plots of molar extinction coefficient, ε, normalized to the absorbance at 410 nm as a function of wavelength are shown for all five blocked oxidized iso-1 proteins in Fig. 2. Between 250 and 600 nm the spectra are similar in shape, although absolute values of ε differ. In addition, Soret bands for Trp-32-SCH₃ and Gln-32-SCH₃ are shifted slightly to the blue and are a little larger than for Lys-32-SCH₃, Leu-32-SCH₃, or Tyr-32-SCH₃. The 695 nm bands (Fig. 2B) have apparent molar extinction coefficients whose values (before background subtraction) follow the order: Lys-32-SCH₃ > Gln-32-SCH₃ > Tyr-32-SCH₃ > Leu-32-SCH₃ ≈ Trp-32-SCH₃. This band originates from ligation of the heme iron (Schechter and Saludjian, 1967) and has been used to assess the conformational integrity of the heme crevice by subtraction of the background absorbance at that wavelength (Kaminsky et al., 1973). If this background subtraction is performed for the iso-1-SCH₃ proteins, their 695 nm bands can be compared. Within experimental error they have approximately equal intensities. Thus, the methionine 85-heme

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**RESULTS**

Iso-1-cytochromes c with Lys-32, Leu-32, Gln-32, Tyr-32, and Trp-32 were prepared from normal and mutant strains of *S. cerevisiae*. These proteins were chemically modified with methyl methanethiosulfonate to replace the sulfhydryl group at Cys-107 with S-SCH₃ (Smith et al., 1975) and thereby prevent dimerization via disulfide linkages at that residue. Rambas et al. (1986) reported that both MMTS-modified and unmodified iso-1 proteins have identical spectral and physical properties. Chemical blocking can therefore provide two advantages for the experiments reported here: first, iso-1 monomers and dimers have distinctly different properties (Bryant et al., 1985). Modifying Cys-107 with MMTS guarantees that the physical measurements obtained are derived from a single monomeric species. Second, when iso-1 proteins are heated,
iron bond appears intact for the oxidized wild type and altered iso-1 proteins at position 32. Furthermore, the heme crevice conformations are probably quite similar as judged by their 695 nm bands, although the overall spectra suggest some possible conformational variation among the proteins.

Guanidine Denaturations—Equilibrium unfolding with guanidine hydrochloride of the oxidized iso-1-SCH₃ proteins was monitored by both CD and fluorescence measurements. The results are presented in Fig. 3 as plots of fraction of unfolding versus denaturant molarity. The molar ellipticity at 220 nm is a function of α-helical structure (Holzwarth and Doty, 1965), while the fluorescence intensity obtained by excitation at 292 nm reflects the degree of Trp-64 fluorescence quenching by the heme group (Bryant et al., 1985), and also indicates the overall protein conformation (Tsong, 1974). For both types of data the primary unfolding transitions for normal and mutant cytochromes occur in a single cooperative event and, within experimental error, the transition midpoints for each protein are independent of method. However, the plots derived from CD and fluorimetry differ in two ways: first, the CD plots all show a pre-equilibrium completed by 0.2 M guanidine hydrochloride. As the iso-1-SCH₃ proteins are initially titrated with Cl⁻ ions, the proteins fold up (Tsong, 1975). Apparently Cl⁻ ions stabilize α-helices at pH 7, but the effect is too small to be observed fluorimetrically. Second, the plots from both methods for each protein are not superimposable. Clearly the slopes in the cooperative region differ. In a pure two-state transition the observed degree of unfolding is independent of experimental technique, even if the observables measure effects derived from different properties of the protein (Lumry et al., 1966). Thus, the transitions shown in Fig. 3 are multistate equilibria and involve a population of significant transitory intermediates. This is most obvious for Gln-32-SCH₃. We therefore present “two-state” limiting energy values as a quantitative parametric measure of conformational stability, without implying any mechanistic significance.

Table I gives calculated parameters for guanidine denaturation of normal and altered iso-1-SCH₃ proteins obtained from linear plots of In K,(Gdn) versus [Gdn] over the range 0.8 ≤ [Gdn] ≤ 0.2. Free energy changes of unfolding in the absence of denaturant, ∆G°, are also given in Table I for both experimental techniques and, except for Gln-32-SCH₃, are the same within experimental error for each protein. Unfolding free energy changes depend on the cooperativity of the transition and −RT∂∆G°/∂T values for Gln-32-SCH₃ differ by 45%. The orders of stability derived from CD and fluorescence ∆G° values are Lys-32-SCH₃ ≈ Leu-32-SCH₃ ≈ Gln-32-SCH₃ (CD) and >Gln-32-SCH₃ (fluorescence) > Tyr-32-SCH₃ ≈ Trp-32-SCH₃. The cooperativities shown in Table I roughly follow the orders of stability obtained from ∆G° values.

Thermal Denaturations—Plots of absorbance at 287 nm normalized to that at 0 °C versus temperature are shown in Fig. 4 for oxidized iso-1-SCH₃ proteins having altered residues at position 32. Tyrosine and tryptophan residues absorb at that wavelength; as the tyrosines are increasingly exposed to solvent by thermal unfolding, their molar extinction coefficient decreases (Donovan, 1973). Except for Gln-32-SCH₃, all the melting curves shown in Fig. 4 consist of a single cooperative transition with positively sloping upper and lower base lines. The thermal profile of Gln-32-SCH₃ differs from the others only in its upper base line, which rises and slowly levels off (especially above 70 °C) as the protein aggregates. The hypochromicity, H, of these absorbances versus temperature plots is

$$H(287\ \text{nm}) = \frac{A(0^\circ) - A(70^\circ)}{A(0^\circ)},$$

where A(0°) and A(70°) are 287 nm absorbances at 0 and 70 °C, respectively. For the iso-1-SCH₃ having various residues at position 32, H(287 nm) varies by greater than 2-fold and depends significantly on the upper base-line slope.

Table II indicates calculated thermodynamic parameters for thermal unfolding of altered iso-1-SCH₃ proteins. The order of stability as judged by either the denaturation mid-point, Tₘ, or ∆ΔH° values is Lys-32-SCH₃ ≈ Leu-32-SCH₃ > Gln-32-SCH₃ > Tyr-32-SCH₃ ≈ Trp-32-SCH₃. This is the same order given by ∆G° values derived from fluorescence measurements of guanidine denaturation. The enthalpy changes for thermal denaturation are at least as great for Leu-32-SCH₃ and Gln-32-SCH₃ as for the normal protein, while those for the Tyr- and Trp-substituted proteins are clearly lower. These ∆H° values reflect the transition widths shown in Fig. 4: ∆H° decreases for a broader transition with more intermediates (Lumry et al., 1966). ∆S° values follow enthalpy changes, presumably due to enthalpy-entropy compensation. Although plots of ln K(T) versus 1/T for the normal and altered iso-1-SCH₃ appear linear in the transition region, their curvatures can be so gentle that, over the narrow transition temperature range, they are not detectable. If these graphs had been extended over a wider temperature range, as for example in Hawkes et al. (1984), any curvature present implying a heat capacity change would be visible. ∆G° values
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1.0

FIG. 3. Fraction of unfolding, \( f_\alpha \) versus guanidine hydrochloride concentration derived from CD (C) and fluorescence (□) data for A, Lys-32-SCH\(_3\); B, Leu-32-SCH\(_3\); C, Gln-32-SCH\(_3\); D, Tyr-32-SCH\(_3\); and E, Trp-32-SCH\(_3\). Errors in \( f_\alpha \) are estimated to be ±0.08 \( f_\alpha \) units.

TABLE I

| Guanidine denaturation parameters for normal and altered iso-1-cytochromes c-SCH\(_3\) |
|---|---|---|---|---|---|
| Parameters were calculated according to Schellman (1978) as described under "Materials and Methods." Estimated errors are: \( C_m \), ±0.1 mol/liter; \( \Delta G^\circ \), ±0.5 kcal/mol; \(-RT\Delta S^\circ\), ±0.5 kcal-liter/mol. |
| CD Fluorescence CD Fluorescence CD Fluorescence |
| mol/liter kcal/mol kcal-liter/mol |
| Lys-32-SCH\(_3\) 0.88 0.93 2.9 2.8 3.3 3.0 |
| Leu-32-SCH\(_3\) 0.81 0.72 2.7 3.0 3.3 4.1 |
| Gln-32-SCH\(_3\) 0.71 0.73 3.0 1.7 4.3 2.4 |
| Tyr-32-SCH\(_3\) 0.56 0.67 1.2 0.7 2.1 1.1 |
| Trp-32-SCH\(_3\) 0.52 0.56 1.2 0.8 2.3 1.5 |

at 30 °C listed in Table III were therefore calculated using the experimentally measured \( \Delta C_P^\circ \). The large uncertainties in \( \Delta H^\circ \) and \( \Delta S^\circ \) for Gln-32-SCH\(_3\) arise from difficulty in defining the post-transition base line due to aggregation above 50 °C.

DISCUSSION

We have chosen to measure the relative stabilities of altered iso-1-cytochromes c-SCH\(_3\) having four different substitutions at position 32 by monitoring their chemical and thermal denaturations. The resulting data suggest the unfolding equilibria are multistate, consistent with the implications from thermal and chemical denaturation of horse heart cytochrome c (Tsong, 1975). Because the experimental methods employed here cannot identify transition intermediates, the data were analyzed with a two-state model. As long as intermediate...
states are kinetically transitory and in low concentrations, a two-state model closely approximates the unfolding equilibria (Zuniga and Nall, 1983; Lumry et al., 1966). Microequilibria involving intermediates do not shift transition midpoints but rather broaden transitions in both the thermal and chemical denaturations of iso-1-SCH₃. Application of two-state models to these thermal and chemical multistate equilibria therefore decreases ΔHό and ΔSό, and ΔGό values, respectively, relative to the ideal case.

Because the stabilities reported here were measured for comparison of the five proteins, it is only necessary that our data set be self-consistent. However, comparison of the iso-1-SCH₃ data presented here with literature values for the modified and unmodified protein verifies that our experimental and analytical procedures are acceptable. Ramdas et al. (1986) reported C₆ and ΔG₆ values for iso-1-SCH₃ from fluorescence measurements within experimental error of those shown in Table I. ΔG₆ obtained by Bryant et al. (1986) for unmodified monomeric iso-1 is in excellent agreement with our value. The corresponding numbers published by Polastri et al. (1976) describe a much more stable protein than ours; we do not understand this discrepancy. The Tₘ for iso-1-SCH₃ listed in Table II is similar to, but slightly lower than that found by Nall,⁵ although our ΔH₂₀ and ΔS₂₀ agree very closely. Differences between our data and literature stabilities presumably depend on the quality of the protein extraction, attention to experimental details, and, most importantly, definition of upper base-line slopes.

Free energy changes associated with guanidine and thermal unfolding of the altered iso-1-SCH₃ differ for several reasons:

⁵ B. Nall, personal communication.
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up to 13 °C and 21 kcal/mol, respectively, from the normal (Hawkes et al., 1984). Because Lys-32 in iso-1 is exposed to solvent (Louve et al., 1988), mutation of surface residue arginine 96 to histidine in T4 lysozyme is both relevant to our work and interesting in that the Tm fell 14 °C (Grutter et al., 1979). These examples demonstrate a range of stability changes and demonstrate that small structural changes can significantly affect physical properties of proteins.

Hydrogen bonding, electrostatic attractions, hydrophobic interactions, backbone conformational entropy, pH effects, disulfide bonds, and the surface tension effect all participate in the free energy stabilization of proteins (Cantor and Schimmel, 1980; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a, 1982b, 1983). The overall stabilization, a delicate balance of large opposing free energies, is usually small. Similarly, each of these factors acts on every residue to provide a net stabilizing contribution to the protein as a whole. From thermodynamic data alone it is difficult to separate the effect on a single residue of each individual force from all the others.

A brief exploration of how several of these forces may determine the relative stabilities of the normal and altered iso-1-SCH3 follows.

Intraresidue and short range interactions are primary contributors to the total energy of a protein in its native form and determine the preferred conformational states of its residues (Nemethy and Scheraga, 1977). The local conformation of an amino acid residue can be represented by its (ϕ, ψ) dihedral angles (Ramachandran and Sasisekharan, 1968). X-ray crystallography has shown that the conformations of both Lys-32 in yeast iso-1-ferricytochrome c and the homologous Lys-27 in tun ferricytochrome c are quite similar (Louve et al., 1988). For Lys-27 in the tuna protein these measured dihedral angles, corresponding to outer and inner molecules in the crystal, are (−105, −132) and (−112, −129), respectively (Takano and Dickerson, 1981). A Ramachandran plot of Lys (ϕ, ψ) dihedral angles from 20 protein crystallographic structures indicates this particular (ϕ, ψ) pair is uncommon among lysines in this protein data set (Nemethy and Scheraga, 1977). The probability of observing this lysine conformation is therefore low for both tuna and yeast ferricytochrome c, thus suggesting that its energy, relative to the more frequently observed lysine conformations, is high.

The free energy change of unfolding measures the difference in stability between the initial and final states of denaturation. A reasonable assumption is that the final unfolded conformation and also the final absolute energy in either type of denaturation are the same for both wild type and altered iso-1-SCH3. CD spectra and molar ellipticities at 220 nm for the different measured stabilities of the altered proteins. These radii of gyration might thus qualitatively explain the relative Cm and Tm values for the altered proteins at position 32. However, stereoscopic drawings of iso-1 seem to show that any of these 4 residues can easily replace the Lys-32 side chain (Louve et al., 1988).

The thermodynamic data presented here have implications for understanding biological systems. First, Lys-32 and Pro-76 are evolutionarily retained residues in the class of typical cytochromes c. Replacement of Pro-76 in iso-1-SCH3 with valine, threonine, or isoleucine followed by guanidine unfolding decreased both ΔG° and Cm values relative to the wild type (Ramdas et al., 1986). When these altered proteins were thermally denatured, the observed Tm values were at least 15 °C lower than that for iso-1-SCH3. In contrast, Leu-32-SCH3 is at least as stable as Lys-32-SCH3 with respect to both chemical and thermal denaturation. This, residues substituting for conserved amino acids do not necessarily destabilize a protein. Another force, alone or in combination with the effects of thermodynamic instability, must therefore be responsible for the selective pressure to retain lysine at position 32.

Second, in vivo activities for altered iso-1-cytochromes c can be estimated by growth in lactate medium (Schweingruber et al., 1978, 1979). Table III lists relative growth rates for a set of isogenic yeast strains having iso-1-cytochromes c altered at position 32 and thermal free energy changes of unfolding, ΔG°, at 30 °C for the corresponding blocked proteins. Since the non-denaturing intracellular iso-1 chemical environment is the same in any of the mutant strains, the primary factor influencing protein stability in vivo should therefore be growth temperature. As shown in Fig. 4, by 30 °C several of the proteins are clearly within the cooperative unfolding transition. The thermal instabilities of the altered cytochromes may therefore reflect the differential in vivo protein conformations of the mutant strains and hence their relative in vivo activities. Also, as shown in Fig. 4, the fraction of unfolding below 25 °C is 0.0 for each of the altered proteins. This may explain why the in vitro enzymatic properties determined at 15 and 25 °C by Das et al. (1988) are
normal or near normal. Table III shows reasonable correlation between in vivo activities and in vitro thermal stabilities at 30 °C. Cm, ΔG°, and Tm values in Tables I and II also parallel relative growth rates of Table III, again suggesting specific measurements of unfolding can estimate in vivo iso-1-cytochrome c activities.

Summary.—Iso-1-cytochromes c having Leu-32, Gln-32, Tyr-32, and Trp-32 replacements for Lys-32 were extracted from strains of S. cerevisiae, chemically modified at Cys-107, and guanidine and thermally unfolded in multistate equilibrium. Although the unfolding free energy changes for each protein depend on the denaturation method, the relative orders of stability do not: Lys-32-SCH3 > Leu-32-SCH3 > Gln-32-SCH3 > Tyr-32-SCH3 > Trp-32-SCH3. Replacement of Lys-32 resulted in a range of stabilities suggesting that small structural changes may significantly affect protein stability. The results indicate that, within the class of normal cytochrome c structures, replacement of an evolutionarily retained residue by another does not necessarily destabilize the protein. Also, in vivo iso-1 specific activities correlate with in vitro thermal stabilities at 30 °C, the optimum growth temperature for yeast.

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